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Nanofluidic Pre-Concentration Devices for Enhancing the Detection Sensitivity and Selectivity of Biomarkers for Human Performance Monitoring

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14. ABSTRACT
To effectively monitor human performance biomarkers, we applied electrokinetic preconcentration technique of the biomarkers onto patterned graphene-modified\ electrodes in a nanochannel by frequency-selective dielectrophoresis using the molecular dam operation. Low copy numbers of human performance biomarkers can be enriched several orders of magnitudes and detected in tens of seconds. Employing this methodology and electrochemical detection, we have demonstrated the ultrafast and ultrasensitive detection of human performance biomarkers, such as neuropeptide NPY and OXA at pM levels, cortisol at detection limit of 30 pg/ml, all can be detected in 5 minutes, in addition to cancer biomarker prostate specific antigen at 1 pg/ml in 30 seconds. All these are achieved from subnanoliter samples, with sufficient signal sensitivity to avoid from high levels of interferences within biological matrices. We obtained kinetic curves for titration of various target concentrations, developed testing nanoslit devices for spiked samples, and tested nanoslit devices for clinically relevant samples with nanoscale DEP molecular dam target enrichment or capturing. We advanced the field by first ever applications of nanoscale dielectrophoretic molecular dam as sample enrichment technique combining grapheme-modified electrode for electrochemical detection for ultrafast and ultrasensitive human performance biomarkers monitoring. Given the high sensitivity of the methodology within small volume samples, we envision its utility toward off-line detection from droplets collected by microdialysis for the eventual measurement of human performance biomarkers at high spatial and temporal resolutions.

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Summary:

To effectively monitor human performance biomarkers, we applied electrokinetic preconcentration technique of the biomarkers onto patterned graphene-modified electrodes in a nanochannel by frequency-selective dielectrophoresis using the molecular dam operation. Low copy numbers of human performance biomarkers can be enriched several orders of magnitudes and detected in tens of seconds. Employing this methodology and electrochemical detection, we have demonstrated the ultrafast and ultrasensitive detection of human performance biomarkers, such as neuropeptide NPY and OXA at pM levels, cortisol at detection limit of 30 pg/ml, all can be detected in 5 minutes, in addition to cancer biomarker prostate specific antigen at 1 pg/ml in 30 seconds. All these are achieved from subnanoliter samples, with sufficient signal sensitivity to avoid from high levels of interferences within biological matrices. We obtained kinetic curves for titration of various target concentrations, developed testing nanoslit devices for spiked samples, and tested nanoslit devices for clinically relevant samples with nanoscale DEP molecular dam target enrichment or capturing. We advanced the field by first ever applications of nanoscale dielectrophoretic molecular dam as sample enrichment technique combining grapheme-modified electrode for electrochemical detection for ultrafast and ultrasensitive human performance biomarkers monitoring. Given the high sensitivity of the methodology within small volume samples, we envision its utility toward off-line detection from droplets collected by microdialysis for the eventual measurement of human performance biomarkers at high spatial and temporal resolutions.

Introduction:

A methodology for the real-time assessment of a diverse set of performance biomarkers, which are indicators of fatigue, vigilance, and stress, is currently being undertaken by the 711th Human Performance Wing at the Wright Patterson Air Force Base to assess the preparedness of its personnel for various missions. The importance of this work is to overcome the current sensor platform which is unable to sensitively monitor these biomarkers within real biological fluid media such as saliva and blood, due to the presence

of interfering proteins at far higher levels, so that their levels can eventually be monitored in biological fluids in real time. The specific aims include (1) the development of nanofluidic slit (nanoslit) sensor devices with target enrichment or capturing by dielectrophoresis (DEP), in the context of nanoscale molecular dam operation, for human performance relevant biomarkers and (2) the detection of the pertinent performance biomarkers neuropeptide-Y (NPY) and Orexin A (OXA), cancer biomarker prostate-specific-antigen (PSA) and metabolite cortisol in nanoslit devices using fluorescence imaging and grapheme-modified electrode for electrochemical detection. The ultimate goal of the work is to develop an integrated multi-modal sensor platform for multiple biomarker target detection for effectively monitoring the mission preparedness of the Air Force personnel.

Experiments:

1. Chemicals and Instrumentation

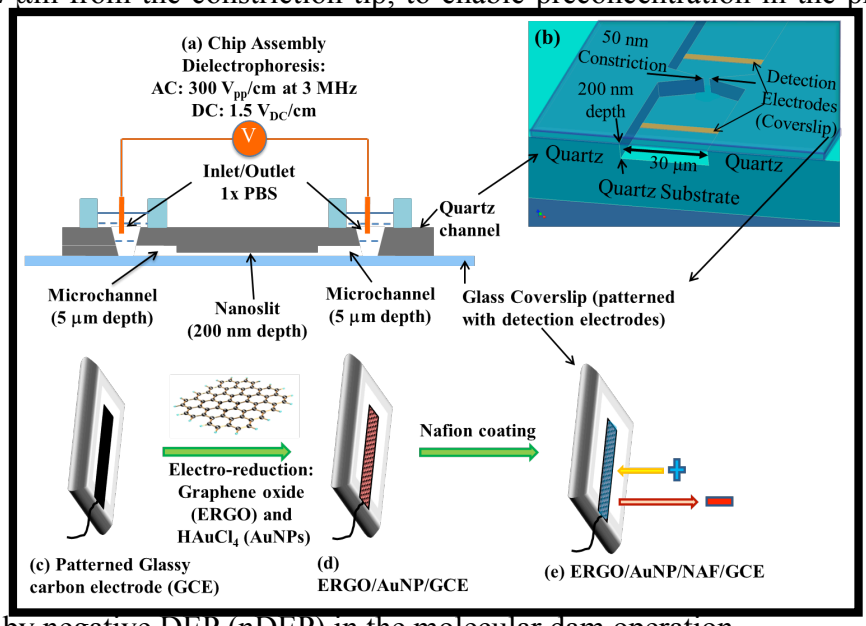
All chemicals were of A. R. grade and were used as received without any further purification. NPY, NPY 18-36 and OXA were obtained from Phoenix Pharmaceuticals, Inc. Graphite powder and chloroauric acid ($HAuCl_4$) were purchased from Sigma Aldrich. Nafion (NAF, 1100EW, 5 wt% aqueous alcoholic solution, Aldrich) was prepared as 0.1% solution by dilution with ethanol. All solutions were prepared using double distilled water of specific conductivity (0.3-0.8 μS/m). Phosphate buffer solution (PBS; 0.1 M, pH 6.0) was employed as a supporting electrolyte. NPY, NPY18-36 and OXA were determined in artificial cerebrospinal fluid, sweat, saliva and blood serum samples (Sigma Aldrich).

All voltammetric, chronocolometric and electrochemical impedance spectral (EIS) measurements were performed on Solartron analytical, Electrochemical Work Station, model Modulab 2101A. An Ag/AgCl, 3M KCl and a platinum electrode were used as reference and counter electrodes respectively. Glassy carbon electrode (GCE), AuNP/NAF/GCE, ERGO/NAF/GCE and ERGO/AuNP/NAF/GCE were employed as working electrodes. The pH measurements were performed using Mettler Toledo FE20 pH meter. XRD analysis was carried out on an X-ray diffractometer (Shimadzu 7000S, Shimadzu Analytical, Japan). The UV–visible spectroscopy was carried out on a Shimadzu spectrophotometer with samples in a quartz cuvette operated from 200 to 800 nm.

2. Microfluidic chip assembly

Figure 1 shows the schematic process for assembly of the microfluidic chip used in this work. It consists of a quartz substrate nanofabricated with the channel structures and bonded to a glass cover-slip that was microfabricated with electrochemical detection electrodes. As per the cross-sectional view in **Figure 1(a)**, the quartz substrate has an inlet and outlet leading to microchannel reservoirs of 5 μm depth and 750 μm width, which lead to several nanochannels of 200 nm depth and 30 μm width. The vertical constriction in the depth direction leads to a slit-shaped nanochannel, which contains lateral constrictions down to 50 nm in width, arranged ~100 μm from the interface of the nanochannel to the micro-channel, as per the top view in **Figure 1(b)**. The procedure for nanofabrication of channels within quartz was extensively described in prior work.[35, 36] The glass cover-slip was patterned with the electrochemical detection electrodes of 30 μm width and running length-wise across the cover-slip, which was fabricated as per **Figure 1 (c-e)**. Briefly, a thin layer (<0.1 μm) of positive photoresist (AZ 701 MiR, Germany) was patterned by standard photolithography methods of soft bake and exposure using a broadband UV source (Karl Suss MJB3) to yield

patterned resist after development. The patterned resist was converted to a glassy carbon layer by standard pyrolysis methods, as described previously using a 1000°C furnace with an inert atmosphere for 60 min. The patterned chip was sonicated in distilled water for about 30 s, and allowed to dry under infrared lamp. GO was synthesized directly from graphite by Hummers method (J. Am. Chem. Soc. 1958, 60, 1339-1339). The synthesized graphite oxide powder was exfoliated in doubly distilled water by ultrasonication for 2 h to form homogeneous GO dispersions with a concentration of 1.0 g/L. Electro-reduction of graphene oxide (ERGO) on the patterned GCE surface was conducted as per prior reports (Microchim Acta 2012, 177, 325–331). In brief, 7 µL of the exfoliated GO dispersion was immersed into 20 mM KH_2PO_4 solution, and a cathodic potential of -0.8 V was applied for 5 min using a potentiostat to obtain ERGO/GCE. For the modification of ERGO with AuNPs by electrodeposition, a constant potential of -0.8 V was applied in a de-aerated precursor solution of 0.5M H_2SO_4 containing 1mM $HAuCl_4$ for an optimal time of 60 s. After electrochemical co-reduction, the coverslip surface was washed with doubly distilled water and dried under I. R. lamp. NAF was delivered selectively through a nanopipette by drop casting under a stereomicroscope on to the graphene modified GCE surface patterned on the glass cover-slip, followed by sonication and drying. Following the microfabrication, the cover-slip was bonded to the quartz microchannel substrate using procedures from our prior work after ensuring that the edge of graphene-modified electrode was aligned to be within a distance of ~2 µm from the constriction tip, to enable preconcentration in the proximity of the electrodes by negative DEP (nDEP) in the molecular dam operation.



3. Analyte Preconcentration and Detection

The negative DEP behavior of NPY under an AC field of 300 V_{pp}/cm at a frequency of 3 MHz was driven by a standard function generator (Agilent 3220) and voltage amplifier (FLC). To enhance transport of NPY towards the constriction, an additional DC field of 1.5 V/cm was utilized. The applied field (AC to DC ratio and frequency) was optimized to enhance nDEP preconcentration and cause spatial localization in a narrow region away from the constriction tip by using fluorescently labeled NPY (supplier) and inverted microscopy to image the preconcentration profile on an EMCCD (Hammatsu). Following optimization, the electrochemical analysis by Differential Pulse Voltammetry (DPV) was carried out using the graphene-modified electrodes. Preconcentration studies based on electrochemical

Figure 1. Cross-section (a) and Top-view (b) of the chip assembly with a micro-channel (5 µm depth) and several slit shaped nano-channels (200 nm depth) containing ~50 nm lateral constrictions fabricated on a quartz substrate bonded to a glass coverslip with patterned graphene modified electrochemical detection electrodes as per: (c)–(e).

adsorptive accumulation were carried out for all the neuropeptides studied herein: NPY, NPY 18-36 and OxA. For this purpose, an accumulation potential of -0.7 V was applied to the graphene-modified electrode for various set time intervals (10 s to 450 s), followed by DPV by scanning the potential towards the positive direction from +0.4 to +1.0 V, using a step potential of 5 mV and modulation amplitude of 50 mV. Renewal of the electrode surface was accomplished by soaking the modified electrode into the supporting electrolyte and cycling the potential between -1.5 V and +0.6 V (vs. Ag/AgCl) in PBS (pH 6.0) buffer solution five times before use so as to renew the electrode surface. The cyclic voltammetric experiments were carried out by scanning the potential from 0.3 V to +0.9 V. Double potential step chronocoulometry was carried out with a pulse period of 5 s from +0.45 V to +0.95 V *vs*. Ag / AgCl.

Modification of secondary PSA antibody for sandwich assay. The sandwich assay for electrochemical detection was performed using Alkaline phosphatase (ALP) tagged to the secondary PSA antibody by glutaraldehyde coupling, for enabling the selective catalysis of α-naphthyl phosphate (NPP) to the electroactive α-naphthol (NP) product, as per the detection scheme in Figure 1. For ALP tagging of anti-PSA, an appropriate amount of ALP solution was transferred to an Eppendorf tube and mixed with anti-PSA in deionized water. Next, 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) was added to the solution. The resulting mixture was incubated with shaking (120 rpm) at room temperature for 10 min, and then incubated for 4 hours in the dark. Finally, 1 M monoethanolamine solution was added to the mixture, which was subsequently incubated with shaking (100 rpm) for 2 h at room temperature. The mixture was dialyzed against PBS solution at 4 °C overnight. The dialysis product, PSA antibody-ALP conjugate, was then transferred to an Eppendorf tube and mixed with an equal volume of glycerin and 1% BSA, for storage at 0 °C, prior to the experiments.

Cortisol, Triamcinolone, Estradiol, Testosterone and Progesterone were procured from Sigma-Aldrich, USA. Cortisol aptamer was purchased from IDT with a 5′-thiol modification and purified by HPLC sequence (Martin et al., 2014). The final sequence is:

GGAATGGATCCACATCCATGGATGGGCAATGCGGGGTGGAGAATGGTTG
CCGCACTTCGGCTTCACTGCAGACTTGACGAAGCTT

The aptamer-functionalized nanoparticles were suspended in 200 μL of 1X PBS at pH 7.5. For passivating rest of the AuNP surface with mercaptohexanol (MCH), 28 μL of 4 μM MCH was mixed with 1 mL of 10 nM aptamerfunctionalized AuNPs, followed by vortexing for 40 s, and washing with 1 mL ethyl acetate (vortexing for 40 s and removing the organic phase layer) and multiple wash steps in PBS to obtain aptamer- functionalized AuNPs with MCH passivation. For pre-binding Triamcinolone to these aptamer- functionalized AuNPs, Triamcinolone was first dissolved in ethanol and re-suspended with aptamer- functionalized AuNPs in the binding buffer composed of: 100 mM Tris–HCl at pH 8, 200 mM NaCl, 25 mM KCl, 10 mM MgCl2 and 5% ethanol. The mixture was incubated at 4 °C overnight to enable complete pre-binding of Triamcinolone to the cortisol aptamer. The excess unbound Triamcinolone supernatant was removed from the aptamer-functionalized AuNPs by multiple wash steps and filtration using Amicon ultrafilters (EMD Millipore). The complete removal of any non-specifically bound Triamcinolone from aptamer-functionalized AuNPs was confirmed by voltammetry on the filtered nanoparticles, to check for absence of the characteristic peak associated with electrochemical reduction of Triamcinolone. Following this, the passivated aptamer- functionalized AuNPs pre-bound with Triamcinolone were stored in the binding buffer at 4 °C, under dark conditions, until they were ready for use

within the cortisol assay.

4. Analytical determination in biological matrices

Neuropeptides detection. Analysis of NPY, NPY18-36 and OXA was carried out in spiked artificial cerebrospinal fluid, sweat, saliva and blood serum samples. Sample analysis was performed for determination of NPY, NPY18-36 and OXA by spiking standard solutions of these neuropeptides into pH 6.0 PBS so as to obtain an optimum concentration. For the determination of neuropeptides, no pre-treatment step was carried out. The cleaning of all the samples was done by filtering through a 0.22 μm PVDF syringe filter (Millex, Millipore Corporation) before voltammetric measurements. The results obtained were then validated by HPLC-MS method.

PSA detection. Three types of experiments were performed to compare the binding kinetics of PSA with immobilized anti-PSA capture probes on the AuNPs on the ERGO modified GC electrode surfaces using electrochemical detection with: (i) ~1 μL scale droplets defined on SU8 patterned microfabricated cover-slip; (ii) nanoslit device (~0.1 nL) integrated to the microfabricated cover-slip (no nDEP enrichment); and (iii) nDEP enriched PSA within the nanoslit device integrated to the microfabricated cover-slip. For case (iii), the Pt electrodes (Alfa Aesar) within each reservoir were programmed to a sequence of electrical signals. The sequence involves a DC field of 50 V/cm for 10 s to enable electrokinetic filling of PSA (concentration varied from 1 pg/mL – 1 ng/mL) and NPP (100 mM), followed by the time period for which nDEP was conducted using ~200 V_{pp}/cm at 6 MHz with a DC offset of 1.5 V/cm, and then ALP-tagged secondary anti-PSA and BSA were filled in the reservoirs, prior to activation of the last sequence with a DC field of 50 V/cm for 10 s to enable electrokinetic filling into the nanoslit. For case (ii), a pulse-free flow system (neMESYS Syringe pump from Cetoni, Inc.) was used for PSA transport, instead of nDEP enrichment, for gauging the binding kinetics within the nanoslit in the absence of nDEP enrichment. For case (i), electrochemistry was performed inside the ~1 μL droplet. Square-wave voltammetric measurements of α-NP were performed in the pH 6.5 PBS media by scanning from 0.1 to 0.4 VDC, using a 100 Hz frequency; 4 mV step potential; and 25 mV AC amplitude. Voltammetric scans can be completed within a few seconds. For electrochemical analysis within the 1 μL droplet, a short accumulation time (10 s) at a deposition potential of -0.1 V was used to enhance adsorption. Standard deviations (95% confidence levels) were based on three different electrochemical measurements at each time point for each of the PSA concentration levels reported herein.

Cortisol detection. For analysis of cortisol in biological media (serum and saliva), a small volume of cortisol at high concentration was made within he citrate–phosphate buffer and this was spiked into the serum media to result in a net media conductivity of 1.6 S/m, which is necessary for electrochemical analysis. The resulting signals were compared with equivalently diluted cortisol in the citrate–phosphate buffer to ensure similarity. For validating the aptamer-based electrochemical assay, we compared its quantification of three deidentified serum samples versus a standard ELISA kit (AbCam ab108665) and a radioimmunoassay (MP Biomedicals Santa Cruz, CA) using standard procedures, conducted over three separate sample runs. For the voltammetric detection, high conductivity citrate–phosphate buffer was spiked into each serum samples at levels necessary to cause a net media conductivity of 1.6 S/m, and the samples were quantified by comparing to signals obtained from equivalently diluted cortisol within the 1.6 S/m citrate–phosphate buffer.

Results and Discussion:

We present a microfluidic device methodology to couple the electrokinetic preconcentration of Neuropeptide Y (NPY) with its detection through electrochemical oxidation of the tyrosine moiety on graphene-modified electrodes in a nanochannel. NPY preconcentration was initiated by applying -0.7 V on the detection electrode for an accumulation period of 300 s or by negative dielectrophoretic trapping away from lateral insulator constrictions within the nanochannel using Pt external electrodes at AC fields of 300 V_{pp}/cm at 3 MHz and a DC field of 1.5 V/cm. Under these conditions, NPY was detected at sensitivity levels down to 4 pM within sub-nanoliter sample volumes, after localized preconcentration by electrochemical adsorptive accumulation for 300 seconds or by dielectrophoresis for 10 seconds. The electrochemical adsorptive accumulation for 300 seconds was also applied to preconcentrate and detect OXA at levels down to 22 pM. This detection platform was compatible with biological matrices such as artificial cerebrospinal fluid (CSF), sweat, saliva and serum. Interference effects from dopamine were avoided due to the occurrence of its electrochemical activity at significantly shifted potentials from that of NPY, OXA or NPY 18-36. Interferences from negatively charged molecules, such as ascorbic acid was avoided due to its negative charge at pH 6, since the cation exchanging nafion overlayer on the graphene-modified electrode prevented the adsorption of anionic species. This detection platform in conjunction with microchip capillary electrophoresis separation is applicable to the detection of neuropeptides at high sensitivity levels and within small sample volumes.

Prostate Specific Antigen (PSA) biomarkers can be significantly enriched within just a few seconds (~25-fold preconcentration in just over a second) in regions away from sharp constrictions on a nanoslit device, using a 6 MHz AC field of 200 V_{pp}/cm alongside a 1.5 V/cm DC field. The nDEP enriched PSA biomarkers can effectively bind to anti-PSA capture probes immobilized on the nanoslit device. Comparing the binding kinetics PSA (5 pg/mL – 1 ng/mL range) to anti-PSA in a microdroplet versus a nanoslit device, with and without nDEP enrichment, we show the steep enhancement in signal versus time on the nanoslit platform, with saturated signal levels achieved within just 2 minutes upon nDEP enrichment.

A quantitative aptamer-based detection methodology for cortisol in a microfluidic device that does not require capture probe immobilization on device surfaces, target labeling, or wash steps prior to the electrochemical readout. The aptamer assay exhibited signal linearity over a five-log cortisol concentration range (10 μg/mL to 30 pg/mL), with rapid binding kinetics with cortisol versus with other glucocorticoids, and the kinetics can be sped-up at detection limit cortisol levels, through assay application within a nanoslit geometry. The assay presented no major signal interferences from estradiol, testosterone and progesterone, except for the case of cortisol at its lowest biologically relevant level within serum (0.06 μg/mL) along with progesterone at its highest biologically relevant level within serum (1 μg/mL), wherein an 18% signal rise was apparent. The concentration of cortisol obtained using this aptamer assay correlated well with those obtained from traditional methods, such as ELISA and radiolabeling, while requiring a much shorter assay time (2.5 min for our assay compared to hours for ELISA or radiolabeling) and smaller sample volumes (<1 μL).

Overall, we have demonstrated the ultrafast and ultrasensitive detection of human performance biomarkers, such as neuropeptide NPY and OXA at pM levels, cortisol at detection limit of 30 pg/ml, all can be detected in 5 minutes, in addition to cancer biomarker prostate specific antigen at 1 pg/ml in 30 seconds. All these are achieved from subnanoliter samples, with sufficient signal sensitivity to avoid from high levels of interferences within biological matrices. We obtained kinetic curves for titration of various target concentrations, developed testing nanoslit devices for spiked samples, and tested nanoslit devices for clinically relevant samples with nanoscale DEP molecular dam target enrichment or capturing. We advanced the field by first ever applications of nanoscale dielectrophoretic molecular dam as sample enrichment technique combining grapheme-modified electrode for electrochemical detection for ultrafast and ultrasensitive human performance biomarkers monitoring. Given the high sensitivity of the methodology within small volume samples, we envision its utility toward off-line detection from droplets collected by microdialysis for the eventual measurement of human performance biomarkers at high spatial and temporal resolutions which have not been achieved before.

List of Publications and Significant Collaborations that resulted from your AOARD supported project:

a) Papers published in peer-reviewed journals:

1. P. Teerapanich, M. Pugnière, C. Henriquet , Y. L. Lin , C. F. Chou, T. Leichle (2016). "Nanofluidic Fluorescence Microscopy (NFM) for real-time monitoring of protein binding kinetics and affinity studies", ***Biosensors and Bioelectronics***, http://dx.doi.org/10.1016/j.bios.2016.06.033; July 2016. **(IF: 7.476)**.

Abstract
Kinetic monitoring of protein interactions offers insights to their corresponding functions in cellular processes. Surface plasmon resonance (SPR) is the current standard tool used for label-free kinetic assays; however, costly and sophisticated setups are required, decreasing its accessibility to research laboratories. We present a cost-effective nanofluidic-based immunosensor for low-noise real-time kinetic measurement of fluorescent-labelled protein binding. With the combination of fluorescence microscopy and reversed buffer flow operation, association and dissociation kinetics can be accessed in one single experiment without extra buffer loading step, which results in a simplified operation and reduced time of analysis compared to typical microfluidic immunoassays. Kinetic constants of two representative protein-ligand binding pairs (streptavidin-biotin; IgG/anti-IgG) were quantified. The good agreement of extracted rate constants with literature values and analogous SPR measurements indicates that this approach is applicable to study protein interactions of medium- and high-affinities with a limit of detection down to 1 pM, regardless of the analyte size.

2. A. Rohani, W. Varhue, K.T. Liao, C.F. Chou, N.S. Swami (2016). "Nanoslit design for ion conductivity gradient enhanced dielectrophoresis for ultrafast biomarker enrichment in physiological media", ***Biomicrofluidics*** 10, 033109 (2016); May 2016. **(IF: 3.357)**

Abstract
Selective and rapid enrichment of biomolecules is of great interest for biomarker

discovery, protein crystallization, and in biosensing for speeding assay kinetics and reducing signal interferences. The current state of the art is based on DC electrokinetics, wherein localized ion depletion at the microchannel to nanochannel interface is used to enhance electric fields, and the resulting biomarker electromigration is balanced against electro-osmosis in the microchannel to cause high degrees of biomarker enrichment. However, biomarker enrichment is not selective, and the levels fall off within physiological media of high conductivity, due to a reduction in ion concentration polarization and electro-osmosis effects. Herein, we present a methodology for coupling AC electrokinetics with ion concentration polarization effects in nanoslits under DC fields, for enabling ultrafast biomarker enrichment in physiological media. Using AC fields at the critical frequency necessary for negative dielectrophoresis of the biomarker of interest, along with a critical offset DC field to create proximal ion accumulation and depletion regions along the perm-selective region inside a nanoslit, we enhance the localized field and field gradient to enable biomarker enrichment over a wide spatial extent along the nanoslit length. While enrichment under DC electrokinetics relies solely on ion depletion to enhance fields, this AC electrokinetic mechanism utilizes ion depletion as well as ion accumulation regions to enhance the field and its gradient. Hence, biomarker enrichment continues to be substantial in spite of the steady drop in nanostructure perm-selectivity within physiological media.

3. Y.L. Lin, Y.J. Huang, P. Teerapanich, T. Leichlé, C.F. Chou (2016). "Multiplexed immunosensing and kinetics monitoring in nanofluidic devices with highly enhanced target capture efficiency", ***Biomicrofluidics***, 2016, 10(3), 034114; May 2016. **(IF: 3.357)**

Abstract
Nanofluidic devices promise high reaction efficiency and fast kinetic responses due to the spatial constriction of transported biomolecules with confined molecular diffusion. However, parallel detection of multiple biomolecules, particularly proteins, in highly confined space remains challenging. This study integrates extended nanofluidics with embedded protein microarray to achieve multiplexed real-time biosensing and kinetics monitoring. Implementation of embedded standard-sized antibody microarray is attained by epoxy-silane surface modification and a room-temperature low-aspect-ratio bonding technique. An effective sample transport is achieved by electrokinetic pumping via electroosmotic flow. Through the nanoslit-based spatial confinement, the antigen-antibody binding reaction is enhanced with ~100% efficiency and may be directly observed with fluorescence microscopy without the requirement of intermediate washing steps. The image-based data provide numerous spatially distributed reaction kinetic curves and are collectively modeled using a simple one-dimensional convection-reaction model. This study represents an integrated nanofluidic solution for real-time multiplexed immunosensing and kinetics monitoring, starting from device fabrication, protein immobilization, device bonding, sample transport, to data analysis at Peclet number less than 1.

4. B.J. Sanghavi, J.A. Moore, J.L. Chávez, J.A. Hagen, N. Kelley-Loughnane, C.F. Chou, N.S. Swami (2016). "Aptamer-functionalized nanoparticles for surface immobilization-free electrochemical detection of cortisol in a microfluidic

device", ***Biosensors and Bioelectronics*** 2016, 78, 244–252. 15 April 2016 **(IF: 7.476)**

Abstract

Monitoring the periodic diurnal variations in cortisol from small volume samples of serum or saliva is of great interest, due to the regulatory role of cortisol within various physiological functions and stress symptoms. Current detection assays are immunologically based and require cumbersome antibody immobilization chemistries, thereby limiting the assay versatility, kinetics, and reproducibility. We present a quantitative aptamer-based detection methodology for cortisol that does not require target labeling, capture probe immobilization on the detection surface or wash steps prior to readout. Using a recognition system of aptamer functionalized gold nanoparticles pre-bound with electro-active triamcinolone, the cortisol level is detected based on its competitive binding to the aptamer by following signal from the displaced triamcinolone using square wave voltammetry at patterned graphene-modified electrodes in a microfluidic or nanoslit device. Due to the 3D analyte diffusion profile at the aptamer interface and the ability to enhance the surface area for cortisol capture, this assay shows signal linearity over a five-log analyte concentration range (10 μg/mL to 30 pg/mL) and exhibits rapid binding kinetics with cortisol versus other glucocorticoids, as apparent from the absence of interferences from estradiol, testosterone and progesterone. The assay is carried out within the biologically relevant range for glucocorticoids in serum and saliva matrices, and benchmarked versus ELISA and radioimmunoassays. Based on absence of cumbersome surface immobilization and wash steps for carrying out this assay, its quantitative signal characteristics and its ability to resist interferences from other glucocorticoids, we envision its application towards routine monitoring of cortisol within bio-fluids.

5. B.J. Sanghavi, W. Varhue, A. Rohani, K.T. Liao, L. Bazydlo, C.F. Chou, N. S. Swami (2015). "Ultrafast immunoassays by coupling dielectrophoretic biomarker enrichment on nanoslit molecular dam with electrochemical detection on graphene", ***Lab Chip*** 2015, 15, 4563-4570. **(IF=6.115)**.

Abstract

Heterogeneous immunoassays usually require long incubation times to promote specific target binding and several wash steps to eliminate non-specific binding. Hence, signal saturation is rarely achieved at detection limit levels of analyte, leading to significant errors in analyte quantification due to extreme sensitivity of the signals to incubation time and methodology. The poor binding kinetics of immunoassays at detection limit levels can be alleviated through creating an enriched analyte plug in the vicinity of immobilized capture probes to enable signal saturation at higher levels and at earlier times, due to higher analyte association and its faster replenishment at the binding surface. Herein, we achieve this by coupling frequency-selective dielectrophoretic molecular dam enrichment of the target biomarker in physiological media to capture probes immobilized on graphene-modified surfaces in a nanoslit to enable ultrafast immunoassays with near-instantaneous (< 2 minutes) signal saturation at dilute biomarker levels (picomolar) within ultra-low sample volumes (picoliters). This methodology is applied to the detection of Prostate Specific Antigen (PSA) diluted in serum samples, followed by validation against a standard two-step immunoassay using three de-identified patient samples. Based on the ability

of dielectrophoretic molecular dam analyte enrichment methods to enable the detection of PSA at 1–5 pg mL^{-1} levels within a minute, and the relative insensitivity of the signals to incubation time after the first two minutes, we envision its application for improving the sensitivity of immunoassays and their accuracy at detection limit levels.

6. T. Leichlé and C.F. Chou (2015). "Biofunctionalized Nanoslit Sensors for Wash-Free and Fast Real-Time Sensing with Spatiotemporally Resolved Kinetics", ***Biomicrofluidics*** 2015, 9, 034103 **(Editor's Picks)**. **(IF: 3.357)**

Abstract
We propose biofunctionalized nanofluidic slits (*nanoslits*) as an effective platform for real-time fluorescence-based biosensing in a reaction-limited regime with optimized target capture efficiency. This is achieved by the drastic reduction of the diffusion length, thereby a boosted collision frequency between the target analytes and the sensor, and the size reduction of the sensing element down to the channel height comparable to the depletion layer caused by the reaction. Hybridization experiments conducted in DNA-functionalized nanoslits demonstrate the analyte depletion and the wash-free detection ~10 times faster compared to the best microfluidic sensing platforms. The signal to background fluorescence ratio is drastically increased at lower target concentrations, in favor of low-copy number analyte analysis. Experimental and simulation results further show that biofunctionalized nanoslits provides a simple means to study reaction kinetics at the single-pixel level using conventional fluorescence microscopy with reduced optical depth.

7. K. K. Sriram, C.L. Chang, U. R. Kumar, C.F. Chou (2014). "DNA Combing on Low-Pressure Oxygen Plasma Modified Polysilsesquioxane Substrates For Single-Molecule Studies", ***Biomicrofluidics*** 2014, 8, 052102 **(IF: 3.357)**

Abstract
Molecular combing and flow-induced stretching are the most commonly used methods to immobilize and stretch DNA molecules. While both approaches require functionalization steps for substrate surface and the molecules, conventionally the former does not take advantage of, as the latter, the versatility of microfluidics regarding robustness, buffer exchange capability, and molecule manipulation using external forces for single molecule studies. Here we demonstrate a simple one-step combing process involving only low-pressure oxygen (O_2) plasma modified polysilsesquioxane (PSQ) polymer layer to facilitate both room temperature microfluidic device bonding and immobilization of stretched single DNA molecules without molecular functionalization step. Atomic force microscopy and Kelvin probe force microscopy experiments revealed a significant increase in surface roughness and surface potential on low-pressure O_2 plasma treated PSQ, in contrast to that with high-pressure O_2 plasma treatment, which are proposed to be responsible for enabling effective DNA immobilization. We further demonstrate the use of our platform to observe DNA-RNA polymerase complexes and cancer drug cisplatin induced DNA condensation using wide-field fluorescence imaging.

8. B. Sanghavi, W. Varhue, J. Chávez, C.F. Chou, N. S. Swami (2014). "Electrokinetic preconcentration and detection of neuropeptides at patterned

graphene-modified electrodes in a nanochannel", ***Analytical Chemistry*** 2014, 86 (9), 4120–4125. **(IF: 5.886)**

Abstract

Neuropeptides are vital to the transmission and modulation of neurological signals, with Neuropeptide Y (NPY) and Orexin A (OXA) offering diagnostic information on stress, depression, and neurotrauma. NPY is an especially significant biomarker, since it can be noninvasively collected from sweat, but its detection has been limited by poor sensitivity, long assay times, and the inability to scale-down sample volumes. Herein, we apply electrokinetic preconcentration of the neuropeptide onto patterned graphene-modified electrodes in a nanochannel by frequency-selective dielectrophoresis for 10 s or by electrochemical adsorptive accumulation for 300 s, to enable the electrochemical detection of NPY and OXA at picomolar levels from subnanoliter samples, with sufficient signal sensitivity to avoid interferences from high levels of dopamine and ascorbic acid within biological matrices. Given the high sensitivity of the methodology within small volume samples, we envision its utility toward off-line detection from droplets collected by microdialysis for the eventual measurement of neuropeptides at high spatial and temporal resolutions.

9. K.K. Sriram, J.W. Yeh, Y.L. Lin, Y.R. Chang, C.F. Chou (2014). "Direct Optical Mapping of Transcription Factor Binding Sites on Field-stretched λ-DNA in Nanofluidic Devices", ***Nucleic Acids Research*** 2014**,** 42, e85. **(IF: 9.202)**

Abstract

Mapping transcription factors (TF) binding sites along a DNA backbone is crucial in understanding the regulatory circuits that control cellular processes. Here we deployed a method adopting bioconjugation, nanofluidic confinement and fluorescence single molecule imaging for direct mapping of TF (RNA polymerase) binding sites on field-stretched single DNA molecules. Using this method, we have mapped out five of the TF-binding sites of *E. coli* RNA polymerase to bacteriophage λ-DNA, where 2 promoter sites and 3 pseudo-promoter sites are identified with the corresponding binding frequency of 45% and 30%, respectively. Our method is quick, robust, and capable of resolving protein-binding locations with high accuracy (~ 300 basepairs), making our system a complementary platform to the methods currently practiced. It is advantageous in parallel analysis and less prone to false positive results over other single molecule mapping techniques like optical tweezers, atomic force microscopy, and molecular combing and could potentially be extended to general mapping of protein-DNA interaction sites.

10. L. Lesser-Rojas, P. Ebbinghaus, G. Vasan, M.L. Chu, A. Erbe, C.F. Chou (2014). "Low-Copy Number Protein Detection by Electrode Nanogap-Enabled Dielectrophoretic Trapping for Surface-enhanced Raman Spectroscopy and Electronic Measurements", ***Nano Letters*** 2014, 14(5), 2242–2250. **(IF=13.779)**

Abstract

We report a versatile analysis platform, based on a set of nanogap electrodes, for the manipulation and sensing of biomolecules, as demonstrated here for low-copy number protein detection. An array of Ti nanogap electrode with sub-10 nm gap size function as templates for AC dielectrophoresis-based molecular trapping, hot spots for surface-enhanced Raman spectroscopy as well as electronic measurements, and fluorescence imaging. During molecular trapping, recorded Raman spectra,

conductance measurements across the nanogaps and fluorescence imaging show unambiguously the presence and characteristics of the trapped proteins. Our platform opens up a simple way for multifunctional low-concentration heterogeneous sample analysis without the need for target preconcentration.

11. L. Lesser-Rojas, K. K. Sriram, K.T. Liao, S.C. Lai, P.C. Kuo, M.L. Chu, C.F. Chou (2014). "Tandem array of nanoelectronic readers embedded coplanar to a fluidic nanochannel for correlated single biopolymer analysis", ***Biomicrofluidics*** 2014, 8, 016501. **(IF: 3.357)**

Abstract
We have developed a two-step electron-beam lithography process to fabricate a tandem array of three pairs of tip-like gold nanoelectronic detectors with electrode gap size as small as 9 nm, embedded in a coplanar fashion to 60 nm deep, 100 nm wide, and up to 150 μm long nanochannels coupled to a world-micro-nanofluidic interface for easy sample introduction. Experimental tests with a sealed device using DNA-protein complexes demonstrate the coplanarity of the nanoelectrodes to the nanochannel surface. Further, this device could improve transverse current detection by correlated time-of-flight measurements of translocating samples, and serve as an autocalibrated velocimeter and nanoscale tandem Coulter counters for single molecule analysis of heterogeneous samples.

12. V. Chaurey, A. Rohani, Y.H. Su, K.T. Liao, C.F. Chou, N.S. Swami (2013). "Scaling down constriction-based (electrodeless) dielectrophoresis devices for trapping nanoscale bio-particles in physiological media of high-conductivity", ***Electrophoresis*** 2013, 34, 1097–1104. **(IF=3.303)**

Abstract
Selective trapping of nanoscale bioparticles (size <100 nm) is significant for the separation and high-sensitivity detection of biomarkers. Dielectrophoresis is capable of highly selective trapping of bioparticles based on their characteristic frequency response. However, the trapping forces fall steeply with particle size, especially within physiological media of high-conductivity where the trapping can be dissipated by electrothermal (ET) flow due to localized Joule heating. Herein, we investigate the influence of device scaling within the electrodeless insulator dielectrophoresis geometry through the application of highly constricted channels of successively smaller channel depth, on the net balance of dielectrophoretic trapping force versus ET drag force on bioparticles. While higher degrees of constriction enable dielectrophoretic trapping of successively smaller bioparticles within a short time, the ET flow due to enhanced Joule heating within media of high conductivity can cause a significant dissipation of bioparticle trapping. This dissipative drag force can be reduced through lowering the depth of the highly constricted channels to submicron sizes, which substantially reduces the degree of Joule heating, thereby enhancing the range of voltages and media conductivities that can be applied toward rapid dielectrophoretic concentration enrichment of silica nanoparticles (~50 nm) and streptavidin protein biomolecules (~5 nm). We envision the application of these methodologies toward nanofabrication, optofluidics, biomarker discovery, and early disease diagnostics.

b) Papers published in non-peer-reviewed journals or in conference proceedings:

13. B. J. Sanghavi, , W. Varhue, A. Rohani, J. L. Chavez, C. F. Chou, N. S. Swami* (2014). "Conformation-Selective Enrichment of Aptamer-Bound Neuropeptides By Dielectrophoresis", ***Proc. 18th International Conference on Miniaturized Systems for Chemistry and Life Sciences (Micro Total Analysis Systems* 2014)**, 2393-2395.

14. K.T. Liao, N. Swami, C.F. Chou* (2013). "Rapid Monitoring Low Abundance Prostate Specific Antigen By Protein Nanoconstriction Molecular Dam", ***Proc. 17th International Conference on Miniaturized Systems for Chemistry and Life Sciences (Micro Total Analysis Systems* 2013)**, 1406-1408.

c) Conference presentations:

15. C.F. Chou (2015). "Low-copy number molecular analysis with dielectrophoretic trapping via plasmonic electrode nanogaps", ***2nd International Conference on Enhanced Spectroscopies (ICES 2015)***, October 12th-15th, 2015, Messina, Italy. **(Invited)**

16. C.F. Chou (2014). "Entropy-driven DNA tug-of-war and confinement-induced reptation in 2D slitlike channels", ***American Physical Society March Meeting***, March 3-7, 2014, Denver, Colorado. **(Invited)**

17. C.F. Chou (2014). "Nanofluidic devices and electrode nanogaps for single/complex biomolecule analysis", ***Pioneer Workshop 2014 on Nanopore and Nanofluidics–Physics and application as Biodevices***, Feb. 7-8, 2014, Osaka, Japan. **(Invited)**

18. C.F. Chou*, K.T. Liao, L. Lesser-Rojas, Y.L. Lin (2013). "Biosensing applications of nanoscale molecular traps and dams", ***15th Asian Chemical Congress (15ACC)***, 19-23 August 2013, Singapore. **(Invited)**

d) Manuscripts submitted but not yet published:

1. R. Fernandez, B. Sanghavi, V. Farmehini, J. Chavez, J. Hagen, N. Kelley-Loughnane, C.F. Chou, N. Swami (2016). "Aptamer-functionalized graphene-gold nanocomposites for label-free detection of dielectrophoretic-enriched neuropeptide Y", ***Electrochemistry Communications***, accepted. **(IF: 4.569)**
2. A. Rohani, B.J. Sanghavi, R.E. Fernandez, K.T. Liao, C.F. Chou, N.S. Swami (2016). "Selective biomarker enrichment in physiological media versus interfering species based on electrical double-layer induced polarization", revision submitted to ***Analytical Chemistry***. **(IF: 5.886)**
3. K. K. Sriram, S. Nayak, C.F. Chou, A. Erbe (2016). "10-nm Deep, Sub-Nanoliter Fluidic Nanochannels on Germanium for Attenuated Total Reflection Infrared (ATR-IR) Spectroscopy", submitted to ***Analyst***. **(IF: 4.033)**

e) Provide a list any interactions with industry or with Air Force Research Laboratory scientists or significant collaborations that resulted from this work.

As this project is in collaboration with Prof. Nathan Swami (University of Virginia) and AFRL scientists (Drs. Jorge L. Chávez, Nancy Kelley-Loughnane and Joshua A. Hagen) at the Wright-Patterson Air Force Base, we have constant interactions with the AFRL scientists. These interactions are enforced through the intensive interactions with Dr. Jorge L. Chávez, who has visited my lab in 2012 and 2016 and attended the AOARD-NSC Taiwan workshop in Kenting, Taiwan, 2012, and the 2nd Joint USAF-Korea-Taiwan Nanoscience Program Review in Xitou, Taiwan, 2016. Our significant collaborations from this work are best represented by three joint journal publications and 1 joint conference proceeding listed below:

1. R. Fernandez, B. Sanghavi, V. Farmehini, J. Chavez, J. Hagen, N. Kelley-Loughnane, C.F. Chou, N. Swami (2016). "Aptamer-functionalized graphene-gold nanocomposites for label-free detection of dielectrophoretic-enriched neuropeptide Y", ***Electrochemistry Communications***, accepted. **(IF: 4.569)**
2. B.J. Sanghavi, J.A. Moore, J.L. Chávez, J.A. Hagen, N. Kelley-Loughnane, C.F. Chou, N.S. Swami (2016). "Aptamer-functionalized nanoparticles for surface immobilization-free electrochemical detection of cortisol in a microfluidic device", ***Biosensors and Bioelectronics*** 2016, 78, 244–252. 15 April 2016 **(IF: 7.476)**
3. B. Sanghavi, W. Varhue, J. Chávez, C.F. Chou, N. S. Swami (2014). "Electrokinetic preconcentration and detection of neuropeptides at patterned graphene-modified electrodes in a nanochannel", ***Analytical Chemistry*** 2014, 86 (9), 4120–4125. **(IF: 5.886)**
4. B. J. Sanghavi, , W. Varhue, A. Rohani, J. L. Chavez, C. F. Chou, N. S. Swami* (2014). "Conformation-Selective Enrichment of Aptamer-Bound Neuropeptides By Dielectrophoresis", ***Proc. 18th International Conference on Miniaturized Systems for Chemistry and Life Sciences (Micro Total Analysis Systems* 2014)**, 2393-2395.

DD882: attached.